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RNAi through short interfering RNA (siRNAs) as a Novel Therapeutic Strategy

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ABSTRACT

Since RNA interference (RNAi) was discovered in the late 1990s, it has evolved as a powerful and widely used strategy for the efficient silencing of genes. RNAi relies on the action of small interfering RNAs (siRNAs) which are incorporated into a complex termed RNA-induced silencing complex (RISC) and guide RISC to its cleavage site on the target mRNA. Thus, the efficiency of RNAi *in vitro* and *in vivo* is determined by the efficacy and intracellular presence of specific siRNA molecules. *In vivo*, the delivery of siRNAs is a major obstacle in the development of RNAi-based strategies also for clinical applications. Various approaches have been explored for the administration of RNAi in different pathological disorders. This review highlights criteria for the development of optimal siRNAs as well as strategies for siRNA stabilization and *in vivo* delivery. Different routes of siRNA administration and various siRNA formulations are discussed. The second part of the review provides a comprehensive overview on siRNA-mediated *in vivo* gene targeting in proof-of-principle studies as well as for the treatment of various pathologies including e.g. viral infection, cancer, liver and renal failure, CNS disorders and pathological ocular neovascularization.

Keywords: gene knockdown, gene-targeting, non-viral siRNA delivery, nanoplexes, RNA interference

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INTRODUCTION

The silencing of an endogenous gene through a homologous dsRNA molecule was first described in petunia flowers already in 1990 (Jorgensen 1990; Napoli *et al*. 1990) and it was termed 'co-suppression' or 'post-transcriptional gene silencing'. Only in 1998, Fire *et al*. demonstrated in the nematode *C. elegans* that in the injection of dsRNAs resulted in marked silencing of a gene, which was 10-100-fold higher than the effect of the corresponding antisense RNA (Fire *et al*. 1998). This silencing mechanism, which was subsequently demonstrated to occur in other eukaryotes including mammals (Hammond *et al*. 2000; Hannon 2002), was termed 'RNA interference' (RNAi).

The natural role of this post-transcriptional, sequence-specific and evolutionary conserved gene-silencing mechanism is believed to be a cellular defense against potentially harmful nucleic acids intruding through viral infection or transposons. Studies on the mechanism of RNAi showed that it is based on short double-stranded RNA molecules of about 21-23 nt, termed small interfering RNAs (siRNAs). These siRNAs are generated intracellularly through the cleavage of longer dsRNAs (Hammond *et al*. 2000; Parrish *et al*. 2000; Yang *et al*. 2000; Zamore *et al*. 2000; Elbashir *et al*. 2001a, 2001b) and rely on certain requirements like a monophosphate at the 5' end and a 2-3 nt overhang at the 3' end for enzyme activity (Elbashir *et al*. 2001). Subsequently, the RNase III-type endonuclease Dicer was identified as the enzyme responsible for dsRNA cleavage into siRNAs (Bernstein *et al*. 2001). More importantly, Tuschl and co-workers showed that chemically synthesized siRNA molecules can induce RNAi, thus confirming siRNAs as RNAi effector molecules and establishing siRNA delivery as sufficient for RNAi induction (Elbashir *et al*. 2001).

Gene silencing through RNAi acts primarily through two distinct pathways, i.e. inhibition of translation and mRNA degradation, with the latter being more efficient and thus being in the focus of the development of drugs based on siRNAs (see Dykxhoorn *et al*. 2005 for review). While siRNAs with high or (almost) complete complementarity to their target mRNA direct cleavage and degradation, lesser complementarity leads to inhibition of translation.

For mRNA cleavage, siRNAs are incorporated into a nuclease-containing multiprotein complex termed 'RNA-in-

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duced silencing complex' (RISC) (Hammond *et al*. 2000). Unwinding of the double-stranded siRNA leads to activation of RISC, which is guided to the cleavage position on the target mRNA strand. RISC also contains the endonuclease Argonaute 2 (Ago2) which, upon binding of RISC to its target mRNA based on the sequence complementarity with the activated siRNA, is in sufficient proximity for target mRNA cleavage at the position directed by the guiding siRNA (Liu *et al*. 2004; Rand *et al*. 2004; Rivas *et al*. 2005). Since this cleavage generates unprotected RNA ends, the target mRNA molecule is rapidly degraded through intracellular nucleases, and becomes thus unavailable for translation. On the other hand, RISC is recovered for further mRNA targeting rounds, making RNAi a catalytical process which may account for its high efficacy.

DEVELOPMENT OF OPTIMAL siRNAs

The systematic comparison of the activity of antisense siRNAs and double-stranded siRNAs regarding target position effects, mRNA cleavage fragments and tolerance for mutational and chemical backbone modifications (see below) indicate that both molecules indeed share the same RNAi pathway. Holen et al. have also shown that antisense siRNA leads to maximum depletion of target mRNA expression significantly faster as compared to double-stranded siRNA indicating that the latter indeed enters the RNAi pathway at an earlier stage, and that both molecules compete for the same pathway (Holen *et al*. 2003).

Several studies have allowed the definition of some criteria to identify siRNA sequences with optimal efficacy and high specificity. Some of these rules are rather straightforward and well-known already from other targeting strategies like antisense or ribozyme-targeting, e.g. the test for partial sequence homologies with unintended target genes, and the avoidance of unwanted internal secondary structures within the targeting oligonucleotide and within the mRNA at the target site (Holen *et al*. 2002; Lee *et al*. 2002; Bohula *et al*. 2003; Ding *et al*. 2003; Kretschmer-Kazemi Far *et al*. 2003; Vickers *et al*. 2003; Xu *et al*. 2003). Other guidelines, however, are more siRNA specific and include the optimal length of 19-25 bp, a GC content in the range of 30-52% and the requirement of symmetric 2 nucleotide overhangs at the 3' end. Additionally, an A in position 3 and a G at position 13 of the sense strand, the absence of a C or G at position 19 and, most importantly, a U in position 10 of the sense strand represent specific sequence biases. Some of these guidelines have been connected to a functional asymmetry, leading to an increased rate of guide strand uptake into RISC, and to the cleavage mechanism of RISC. It was also shown that the thermodynamic flexibility of the positions 15-19 of the sense strand correlates with the silencing efficacy and that the presence of at least one A/U base pair in this region improves siRNA-mediated silencing due to a decreased internal stability of its 3′-end (Donis-Keller 1979; Elbashir *et al*. 2001, 2002; Holen *et al*. 2002; Khvorova *et al*. 2003; Schwarz *et al*. 2003; Reynolds *et al*. 2004; Boese *et al*. 2005).

Algorithms based on these guidelines are readily available from various websites allowing the prediction of optimal siRNAs, see e.g. websites from Ambion (www.ambion.com/techlib/misc/siRNA_finder.html), from Dharmacon (http://www.dharmacon.com/sidesign/default.aspx), from the Tuschl lab (www.rockefeller.edu/labheads/tuschl/sirna.html), or from the Whitehead Institute (jura.wi.mit.edu/bioc/sirnaext). The SDS (siRNA Design Software) provided by the Department of Computer Science, The University of Hong Kong (http://i.cs.hku.hk/~sirna/software/sirna.php) is a software tool that helps to design siRNAs by combining different algorithms. However, since these algorithms are still not perfect, several siRNAs need to be tested experimentally in order to identify maximally efficient siRNAs without unwanted side-effects. The aspect of so-called off-target effects, i.e. non-specific silencing of non-target genes, is particularly important since in some cases, regions showing sequence identity with the target gene in only 11-15 contiguous nucleotides were demonstrated to be sufficient to induce RNAi (Jackson *et al*. 2003). Another study reported the downregulation of HIF-1α mRNA due to off-target gene silencing even through a 7 nt motif and showed that this effect depends on the sequence context surrounding the complementary region (Lin *et al*. 2005).

Additionally, the activation of Toll-like receptors (TLRs) of the innate immune system may lead to inflammatory responses through stimulation of inflammatory cytokine release upon systemic administration of siRNAs to mice. This is particularly true for GU-rich sequences (e.g., UGUGU; GUCCUUCAA) and seems to depend on the siRNA amounts and on the mode of siRNA delivery (Sledz *et al*. 2003; Heil *et al*. 2004; Judge *et al*. 2005; Sioud 2005). In fact, it was demonstrated in mice that liposomal delivery of unmodified siRNAs leads to stronger interferon responses as compared to naked siRNAs (Sioud *et al*. 2003; Heidel *et al*. 2004; Ma *et al*. 2005).

STRATEGIES FOR siRNA STABILIZATION

While RNAi is very powerful through the exploration of the natural intracellular machinery, the major bottleneck for the development of RNAi-based therapies is the stabilization and delivery of siRNAs since naked siRNAs are prone to degradation and do not readily penetrate cellular membranes.

Although siRNAs seem to show higher stability in mammalian cells and physiological fluids than antisense ODNs (Bertrand *et al*. 2002), chemically unmodified siRNAs have a short half life of no more than 15 min in serum (Dowler *et al*. (2006) and Aigner *et al*. unpublished data). To increase stability, various modifications at different positions have been incorporated into either strand. To avoid interference with the gene targeting efficacy, however, modifications in the passenger strand, rather than the guide strand, are often preferred. Phosphorothioate modifications significantly improved serum stability and tissue accumulation (Braasch *et al*. 2003, 2004), but have been shown to be problematic with respect to toxicity (Harborth *et al*. 2003). Other backbone modifications include the introduction of 4' thioribose which, after optimization regarding number and position, displayed equal or superior targeting efficacies and increased thermal and plasma stability as compared to non-modified siRNA molecules (Dande *et al*. 2006). Likewise, 2' modifications have been tested for preservation of siRNA activity. The targeting activity was retained in siRNAs upon partial substitution with 2'-fluoro bases (Braasch *et al*. 2003; Harborth *et al*. 2003) or with so-called locked nucleic acids (LNAs) synthesized through introduction of a methylene linkage between positions 2' and 4' (Braasch *et al*. 2003). The incorporation of LNAs led to higher thermal stability of the hybridization of the siRNAs (Braasch *et al*. 2003) and to higher stability and substantially increased halflife in serum (Elmen *et al*. 2005). Likewise, the serum stability of 2'-F-modified siRNAs was increased; *in vivo*, however, upon i.v. injection into the tail vein they did not display prolonged or enhanced targeting efficacy (Layzer *et al*. 2004). On the other hand, 2'-O-Me, especially when introduced into the 5' end of the antisense strand, has been shown to inhibit siRNA silencing activity, probably due to interfering with the integration of the strand into RISC or with the unwinding of the siRNA (Prakash *et al*. 2005). Likewise, a fluorescent chromophore or other mutations abolished gene silencing when conjugated to the 3'-end of the antisense siRNA, but not when coupled to the sense siRNA or to the 5'-end of the antisense siRNA (Holen *et al*. 2002; Amarzguioui *et al*. 2003; Harborth *et al*. 2003; Holen *et al*. 2003). Further improvements regarding plasma stability and siRNA activity were achieved through the combination of multiple chemical modifications like for example 4'-thioribose with 2'-O-Me and 2'-O-methoxyethyl substitutions (Dande *et al*. 2006) or 2'-deoxy, 2'-F and 2'-O-Me (Morrissey *et al*. 2005).

STRATEGIES FOR siRNA DELIVERY

Another key requirement for successful RNAi *in vivo* is the delivery of siRNAs to their target cells, target tissues or target organs. Routes for systemic administration include intravenous (i.v.), intraperitoneal (i.p.) and subcutaneous (s.c.) injection, and local delivery can be achieved by direct injection into a target organ or by topical application to a surface. Furthermore, for pulmonary delivery inhalation, intratracheal or intranasal administration can be used, and direct application into the central nervous system avoiding the penetration through the blood-brain barrier is achieved through intraventricular or intrathecal injection. Most intensively explored have been approaches for topical or local delivery of unmodified siRNAs to easily accessible tissues. Local administration routes include intratumoral, intramuscular or subretinal/intraocular injections, the direct application into the CNS, into the vagina or into subcutaneous tissues as well as intranasal application/intratracheal instillation. Since delivery to other organs and elimination through renal filtration is reduced, siRNA amounts required for local administration are generally rather low and delivery does not necessarily require siRNA modifications or formulations. However, these approaches are often invasive and limited to easily accessible tissues.

It has now become clear that the systemic application of siRNAs will rely on the development of delivery strategies. Based on their previous use in gene delivery, i.e. delivery of therapeutic DNA molecules, viral vectors have been applied to RNAi. Due to their inherent ability to transport genetic material into cells, viral vectors provide high transfection efficacy, and adenoviruses, adeno-associated viruses, baculoviruses, lentiviruses and retroviruses are used. However, limited loading capacities, problems in large-scale production and, most importantly, safety risks due to their inflammatory and immunogenic effects and their oncogenic potential pose severe limitations to their applicability (Donahue *et al*. 1992; Lehrman 1999; Liu *et al*. 2003; Sun *et al*. 2003).

The induction of RNAi without viral vectors through direct delivery of siRNAs or siRNA-expressing plasmid DNAs has been studied *in vivo* using various strategies regarding formulation and administration. Main goals are the cellular delivery of nucleic acids, their efficient extracellular protection against enzymatic or non-enzymatic degradation which is especially a problem in the case of unstable siRNA molecules, and their efficient intracellular release upon endocytosis. Additionally, the absence of immunostimulatory effects and, *in vivo*, low toxicity, low non-specific binding to biological structures and slow renal (the size of siRNAs is below the threshold for glomerular filtration) and hepatic clearance need to be taken into consideration. The successful systemic application of any siRNA-based drug will largely depend on the optimal combination of all these requirements. DNA expression plasmids for RNAi typically encode palindromic hairpin loops containing the desired siRNA sequence. Upon transcription, RNAs fold back forming double-stranded short hairpin RNAs (shRNAs) which are recognized as dsRNAs by Dicer and cleaved into the desired siRNAs (Zhang *et al*. 2003). While the introduction of these DNA expression cassettes leads to a long-lasting and highly efficient gene targeting, safety issues need to be considered when using DNA molecules *in vivo*. In contrast, siRNAs cannot integrate into the genome, are smaller and can be synthesized relatively easily.

While *in vitro* several transfection reagents are available for direct siRNA delivery, the *in vivo* administration of siRNAs is more difficult and requires more sophisticated strategies which are currently under intense research. In principle, approaches include either one or a combination of the following: injection of pure, unmodified or chemically modified siRNAs, physical methods (hydrodynamic injection, electropulsation), encapsulation of siRNAs in liposomes, lipoplexes, cationic lipids, complexation of siRNAs in cationic or other carriers, or chemical coupling of siRNAs to specific carrier molecules.

Hydrodynamic transfection in mice is based on the rapid, high pressure injection of rather large volumes into the tail vein and is mainly used for siRNA delivery into the liver. It was hypothesized that the sharp increase in venous pressure leads to the enlargement of liver fenestrae and to the transient enhancement of membrane permeability of hepatocytes, resulting in siRNA uptake through 'hydroporation' (Zhang *et al*. 2004). Only a few studies have used hydrodynamic transfection for delivery to other organs. Keeping in mind that up to 2 ml are injected into a mouse within seconds, it is obvious that this method is not a reasonable therapeutic approach in humans. Additionally, Zhang *et al*. have shown that hydrodynamic injection also induces a transient irregularity of heart function (Zhang *et al*. 2004).

Liposomes have been employed as non-viral envelopes to increase serum stability through siRNA protection, cellular uptake through endocytosis and serum half-life through reduced renal excretion. Systemic application approaches include intravenous, intraperitoneal and subcutaneous injections; likewise, local or topical delivery through intracardiac, transurethral, intrathecal and intratumoral injection has been described. While previous studies on the *in vivo* application of antisense-ODNs or ribozymes have shown that several liposomal/cationic lipid delivery systems are toxic, some liposomes allow application *in vivo*. Many studies use commercially available liposomal reagents like i-Fect, cytofectin GSV, JetSi, lipofectamine, N-[1-(2,3-Dioleoyloxy)]-N,N,N-trimethylammonium propane (DOTAP) or dioleoylphosphatidylcholine (DOPC). When comparing cationic and neutral liposomes (e.g., DOTAP and DOPC, respectively), it seems that the characteristics of the delivery to various tissues or organs are also determined by the charge of liposomal vesicles (Landen *et al*. 2005). The same may be true for the uptake by macrophages, again determining the efficacy of siRNA delivery to the desired organ (Miller *et al*. 1998). In studies which rely on the detection of labeled siRNAs, another ambiguity is the fact that it is hard to determine whether the presence of an siRNA label always represents indeed the presence of intact siRNA molecules. While many liposome-based strategies may offer promising approaches, the deeper understanding of liposomal delivery of siRNAs will allow improvements regarding efficacy of cellular uptake and intracellular release, increased organ specificity and reduced toxicity.

Like liposomes, various nanoparticles have been used previously for DNA delivery, e.g. atelocollagen, chitosan and polyethylenimine (PEI) (Boussif *et al*. 1995; Ochiya *et al*. 1999). Atelocollagen, which is a collagen fragment obtained from type I collagen of calf dermis by pepsin treatment, is a positively charged molecule with little immunogenicity. Upon mixing with negatively charged siRNAs, complexes are formed based on electrostatic interactions with sizes dependent on the siRNA/atelocollagen ratio. These complexes allow the protection of siRNAs and the sustained release at the injection site.

The deacylated derivative of chitin, chitosan, as well as chitosan-coated polyisohexylcyanoacrylate (PIHCA) nanoparticles have been developed for oligonucleotide delivery (Borchard 2001 for review; Maksimenko *et al*. 2005). More recently, studies have been extended towards PIHCA complexation of siRNAs (Pille *et al*. 2006) (see below).

POLYETHYLENIMINES IN siRNA DELIVERY

Polyethylenimines (PEIs) are synthetic, linear or branched polymers with a broad range of molecular weights (Tang *et al*. 1997; Godbey *et al*. 1999; Bieber *et al*. 2001). They are able to form non-covalent complexes with nucleic acids based on their high cationic charge density which is derived from their protonable amino group in every third position (Boussif *et al*. 1995; Behr 1997). Since nucleic acids are compacted in these complexes ('condensation') which allows their cellular endocytosis, certain PEIs have been introduced as DNA transfection reagents (see Kichler 2004;

Wagner *et al*. 2004 and references therein). While this complexation occurs with many, if not all, PEIs, the transfection efficacy seems to be dependent on the molecular weight and the degree of branching of the PEI, the complex size, the buffer conditions during complex formation as well as on the N/P ratio, which describes the ratio between nitrogen atoms of PEI and nucleic acid phosphates (see e.g. Werth *et al*. 2006). Additionally, transfection efficacies *in vitro* depend on the cell line which also determines the pathway of complex internalization (clathrin-dependent or lipid-raft-dependent endocytosis (von Gersdorff *et al*. 2006)).

One major issue of nucleic acid delivery to cells, beyond the efficient endocytosis of the nanoparticles, is the subsequent intracellular release of the nucleic acids from the endosomes/lysosomes. PEI-based complexes seem to be efficiently released through the 'proton-sponge effect', which postulates enhanced endosomal Cl^- accumulation in, and osmotic swelling of, endosomes/lysosomes due to the H^+-buffering polyamine function of PEI, leading to endosome disruption and enhanced nucleic acid delivery (Boussif *et al*. 1995; Behr 1997). However, some studies have conjugated melittin analogs to PEI as membrane-destabilizing reagent in order to further enhance the transfection efficiency of PEI-based complexes *in vitro* and *in vivo* (Boeckle *et al*. 2005, 2006; Shir *et al*. 2006).

Due to the tight condensation of nucleic acids as well as the efficient uptake of the complexes and their efficient subsequent intracellular release, PEI complexation has been extended towards the delivery of catalytically active RNA molecules. PEI-complexed siRNAs (Urban-Klein *et al*. 2005; Grzelinski *et al*. 2006; Werth *et al*. 2006) or ribozymes (Aigner *et al*. 2002) are protected against enzymatic or non-enzymatic degradation in the presence of serum or RNase A. More importantly, PEI complexed siRNAs are efficiently endocytosed by cells and, upon their subsequent intracellular release, display full targeting efficacy (Urban-Klein *et al*. 2005; Grzelinski *et al*. 2006). Probably this does not apply to all PEIs, since Hassani et al. found that *in vivo* jetPEI, although efficient for delivering nucleic acids to cells, did not permit development of siRNA activity when used to introduce siRNAs into the mouse brain *in vivo* at least at the low dose ranges tested (Hassani *et al*. 2005). On the other hand, complexation of siRNAs has been successfully extended to novel PEIs. Werth *et al*. described the preparation of a low molecular weight PEI through the size exclusion chromatography-based fractionation of commercially available 25 kDa PEI, and *in vitro* data indicated low toxicity, high transfection efficacy and efficient protection of siRNA molecules (Werth *et al*. 2006).

Chemical modifications of PEI have been introduced to improve its biocompatibility and reduce toxicity/immunogenicity, to increase its efficiency for gene delivery through enhanced endocytosis or facilitated intracellular DNA or siRNA release, and to achieve target cell specificity of the PEI complexes. *In vitro* studies have shown that PEGylation, i.e. the coupling of polyethylenglycol (PEG) to PEI, can improve complex solubility and decrease complex aggregation. Furthermore, PEGylation is able to reduce the interaction with proteins and the activation of the complement system, leading to enhanced circulation time in the blood and to improved biocompatibility *in vivo* (for review, see Neu *et al*. 2005 and references therein). Additionally, the covalent coupling of ligands which specifically bind to surface proteins of target cells, e.g. the antigen binding Fab' fragment of the OV-TL16 antibody directed against the OA3 surface antigen, leads to enhanced transfection efficacy in OA3-expressing OVCAR-3 cells (Merdan *et al*. 2003). The systemic *in vivo* application of ligand (transferrin)-PEG-PEI/DNA complexes has been shown to exhibit tumor targeted expression *in vivo* (Kursa *et al*. 2003).

In vitro studies have also demonstrated that the number and length of PEG chains has a strong effect on physicochemical and biological properties of PEG-PEI/siRNA complexes, and that graft densities and chain lengths need to be taken into consideration when identifying optimal PEG-PEIs for siRNA delivery (Mao *et al*. 2006). Taken together, these data indicate that the PEI-mediated, non-viral delivery of siRNAs represents a powerful strategy for gene targeting *in vivo*, and may offer innovative therapeutic avenues in gene therapy. This is particularly true upon chemical modification of the PEIs in order to increase their biocompatibility and reduce their non-specific interactions, and to enhance their target tissue specificity through covalent coupling of tissue-specific ligands. The latter approach leads to the development of double specific compounds combining high target gene specificity (siRNA) and target tissue specificity (modified PEIs) while still allowing systemic delivery.

PROOF-OF-PRINCIPLE STUDIES IN siRNA APPLICATION *IN VIVO*

Several proof-of-principle studies have shown the delivery of siRNAs and the siRNA-mediated downregulation of marker genes in different tissues. In postnatal mice, transgene expression in various organs was inhibited upon hydrodynamic transfection (Lewis *et al*. 2002). More specifically, mice were co-injected with luciferase plasmid and synthetic siRNAs, and already 1 day after injection luciferase expression was inhibited by 80-90% in liver, spleen, lung, kidney and pancreas as compared to control siRNA. It was also shown that the inhibition of target gene expression by siRNAs was dose-dependent. In an EGFP transgenic mouse, efficient silencing was observed in hepatocytes 48 h after injection as determined by fluorescent microscopy (Lewis *et al*. 2002). The delivery of siRNAs into liver and limb grafts through organ-selective, catheter-based rapid injection was exploited using siRNAs targeting green-fluorescence protein (GFP). Transient down-regulation of GFP expression in transgenic mice was observed in liver- and limb-transplantation models (Sato *et al*. 2005). Furthermore, Heidel *et al*. show that naked, synthetic siRNAs can be administered to mice and downregulate endogenous or exogenous target gene expression without induction of an interferon response (Heidel *et al*. 2004). Finally, in a subcutaneous mouse tumor model with melanoma cells stably expressing luciferase, the luciferase expression in metastatic hepatic tumors was significantly reduced upon hydrodynamic siRNA injection (Takahashi *et al*. 2005).

Other proof-of-principle studies focus on the downregulation of reporter genes upon siRNA administration through other strategies. In the same study as described above, direct injection of siRNAs into subcutaneous primary melanoma xenografts, followed by electroporation, resulted in significant reduction of luciferase activity in the stably luciferase expressing tumor cells (Takahashi *et al*. 2005). Likewise, GFP targeting in s.c. HeLa xenografts was achieved by intratumoral injection of cytofectin GSV/siRNAs, but not antisense ODNs (Bertrand *et al*. 2002), or in the developing vascular network of chicken embryos upon intracardiac injection of lipoplexes (Bollerot *et al*. 2006). In green fluorescent protein-transgenic mice overexpressing GFP, intratracheal instillation of GFP siRNA led to markedly reduced GFP expression in the lung, but not in the liver (Lomas-Neira *et al*. 2005; Perl *et al*. 2005). Subretinal injection of siRNAs led to decreased EGFP or VEGF levels in the eyes of mice after virally induced EGFP or VEGF transgene expression, respectively (Reich *et al*. 2003). Intraventricular infusion of specific siRNAs in EGFP overexpressing transgene mice resulted in extensive EGFP knockdown, especially in regions adjacent or dorsoventrally and mediolaterally distant to the infusion site (dorsal third ventricle), with lesser knockdown in more distal regions (Thakker *et al*. 2004). Likewise, luciferase targeting was demonstrated through intra-cerebroventricular injection of JetSi + DOPE-formulated siRNAs (Hassani *et al*. 2005). Interestingly, in this study the comparison with linear polyethylenimine did not show siRNA activity after PEI-mediated delivery within the dose range tested (Hassani *et al*. 2005) which may again indicate an siRNA dose-dependence of targeting efficacy.

The *in vivo* delivery of siRNAs targeting the insulin 2 (Ins2) gene via hydrodynamic tail vein injection led to reduced Ins2 mRNA levels (Bradley *et al*. 2005).

In stably luciferase expressing tumor xenografts, Minakuchi *et al*. showed that site-specific administration of atelocollagen-complexed siRNAs inhibited luciferase expression (Minakuchi *et al*. 2004). Likewise, the targeting efficacy of systemically delivered atelocollagen/siRNA complexes has been assessed. In a mouse model of bone metastatic human prostate cancer cells stably expressing luciferase in mouse thorax, jaws and / or legs, i.v. injection of GL3 luciferase siRNAs complexed with atelocollagen resulted in rapid downregulation of luciferase activity (Takeshita *et al*. 2005).

The use of chemically synthesized delivery vehicles rather than naked siRNAs also allows the generation of more sophisticated delivery devices through coupling of ligands as a targeting moiety in order to increase target gene or target cell specificity. One example is the coupling of an anti-transferrin receptor single-chain antibody fragment (TfRscFv) to a liposomal nanoplex. Upon systemic administration of fluorescein-labeled siRNAs complexed with this immunoliposome, the specific and efficient delivery of the siRNAs into the tumor cells of primary and metastatic tumors in three different mouse tumor models was observed (Pirollo *et al*. 2006). More recently, dual-purpose probes, comprising of magnetic nanoparticles labeled with a near-infrared dye, covalently linked to siRNAs and modified with a membrane translocation peptide for intracellular delivery, were employed for in vivo transfer of siRNAs and the simultaneous imaging of its accumulation in tumors. Upon i.v. injection, GFP silencing was observed (Medarova *et al*. 2007).

ANTIVIRAL TREATMENT

Due to the fact that current antiviral drugs or vaccination strategies provide only limited protection or therapeutic options, many studies focus on the siRNA-mediated targeting of virus-relevant gene products. While RNAi has been shown to efficiently suppress virus replication *in vitro*, more recent studies have explored the potential of siRNAs *in vivo*, aiming at the reduction of virus titers as well as at protective effects regarding pathological symptoms and survival.

Tompkins *et al*. challenged mice with lethal doses of the human influenza A virus. Upon administration of siRNAs targeting highly conserved regions of the viral nucleoprotein or acidic polymerase through hydrodynamic transfection, a significant reduction of lung virus titers in infected mice as well as their protection from lethal challenge was observed. The broad efficacy was demonstrated by the protection against lethal challenge with highly pathogenic avian influenza A viruses of the H5 and H7 subtypes, indicating that induction of RNAi through siRNA delivery may be a promising tool for the control of viral infections (Tompkins *et al*. 2004).

Likewise, Ge *et al*. demonstrated that siRNAs targeting conserved regions of influenza virus genes can prevent and treat influenza virus infection in mice. Here, siRNAs were complexed with polyethylenimine (PEI), and upon slow i.v. injection of small volumes, led to reduced virus production in lungs of infected mice. Since this effect was observed upon administration either before or after virus infection, formulated siRNAs may be useful for therapy as well as for prophylaxis of influenza virus infection in humans (Ge *et al*. 2004). Additionally, Thomas *et al*. demonstrated that the removal of residual N-acyl moieties from commerically available linear 25 kDa PEI increases the efficiency of nucleotide delivery and synthesized several linear, fully deacylated PEIs. Complexation of an siRNA targeting the influenza viral nucleocapsid protein gene with deacylated PEIs resulted, upon systemic delivery, in a 94% reduction of virus titers in the lungs of influenza infected mice. Similar results were seen for targeting luciferase expression in the lung (Thomas *et al*. 2005).

Respiratory syncytical virus (RSV) and parainfluenza virus (PIV) infections were treated through intranasal application of siRNAs. Intranasal instillation of siRNAs in the presence or absence of transfection reagents showed prevention and inhibition of individual as well as joint infection by RSV and PIV, indicating that inhaled siRNAs may offer a fast, potent and easily applicable antiviral therapeutic strategy against respiratory viral diseases in man. The relevance of screening for efficacious siRNAs was underlined by the fact that the degree of protection matched with antiviral activity of the siRNA in cell culture. Furthermore, excess of one siRNA moderated the inhibitory effect of the other when targeting both viruses in a joint infection, which suggests a 'ceiling effect' of maximum RNAi efficacy. This may probably be due to competition of the siRNAs for the RNAi machinery and may represent a limitation for the above-mentioned dose-dependence of siRNA delivery, suggesting rather the use of lower dosages at least for inhalation (Bitko *et al*. 2005). The drug ALN-RSV01 has been introduced by Alnylam Pharmaceuticals for targeting the human respiratory syncytial virus (RSV) after viral infection. It is the first example of an antivirus siRNA-based therapeutic a phase I clinical study.

In another study targeting a virus of particular interest, siRNAs previously employed as inhibitors of severe acute respiratory syndrome (SARS) viruses (SARS corona viruses, SCVs) were evaluated for efficacy and safety in a rhesus macaque (*Macaca mulatta*) SARS model. In either prophylactic or therapeutic regimens, siRNA-mediated anti-SARS efficacy was observed as determined by monitoring SARS-like symptoms, SCV RNA levels and histopathological and immunohistochemical analysis of the lung. While siRNA-induced toxicity was absent, a relief from SCV infection-induced fever, diminished SCV viral levels and reduced acute diffuse alveoli damage was observed (Li *et al*. 2005).

More recently, siRNA-mediated targeting was explored as a potential therapeutic option in ebola virus (EBOV) infection. Targeting of the polymerase (L) gene of the Zaire species of EBOV with a pool of four different L gene-specific siRNAs delivered through complexation with a commercially available polyethylenimine ('*in vivo* jetPEI') led in guinea pigs to decreased plasma viremia levels and partial protection of the animals from death when administered shortly before the ZEBOV challenge. The use of SNALPs as a delivery system was even more efficacious, leading to complete rather than partial protection under the same conditions. Interestingly, one of the four siRNAs alone was equally efficient as the mixture, indicating the absence of an additive effect of the combination of different siRNAs targeting different regions of an mRNA molecule (Geisbert *et al*. 2006). Likewise, in another study, decreased hepatitis B virus (HBV) titers were observed upon siRNA-mediated targeting of HBV RNA. siRNAs were i.v. injected and displayed again a dose-dependent effect which lasted for 7 d. Direct comparison of unformulated with SNALP-encapsulated siRNAs revealed higher efficacy of the latter, correlating with a longer half-life in plasma and liver (Morrissey *et al*. 2005). Hepatitis B virus replication was also inhibited by siRNA-mediated targeting of the surface antigen region (HBsAg). In mouse models relying on the generation of HBV viral particles upon injection of HBV plasmids, injection of siRNAs resulted in a significant reduction of viral transcript, antigen and DNA levels in the livers and sera (Giladi *et al*. 2003; Klein *et al*. 2003).

CANCER

Several studies have focused on the siRNA-mediated downregulation of genes which have previously been demonstrated as cancer-relevant. Primary goals are the reduction or abolishment of tumor growth and tumor metastasis.

Targeting survivin through intratumoral injection of siRNAs prevented growth of bladder cancer xenografts (Hou *et al*. 2006). Intratumoral administration was also em-

ployed for the delivery of atelocollagen-complexed siRNAs targeting luciferase expression in a xenografted tumor. In the same study, in an orthotopic xenograft model of a human non-seminomatous germ cell tumor, atelocollagen-mediated delivery of siRNAs targeting HST-1/FGF-4 led to inhibition of tumor growth (Minakuchi *et al*. 2004). Likewise, atelocollagen/siRNA-mediated VEGF targeting was shown to inhibit tumor growth and tumor angiogenesis in a s.c. prostate carcinoma xenograft model (Takei *et al*. 2004). Furthermore, intratumoral injection of siRNAs targeting the Ewing sarcoma chimeric oncogenic transcription factor EWS-Fli1 led to dose-dependent anti-tumorigenic effects. The comparison between free and encapsulated siRNAs also showed that nanocapsules improved the intracellular penetration of siRNAs (Toub *et al*. 2006). However, apart from the rather limited therapeutic relevance of this approach of direct administration to the tumor, other studies also indicate that the success of i.t. injections can depend on the integrity of the tumor xenografts with some tumor tissues being too solid for injection of sufficient amounts of volume (Hobel and Aigner, unpublished data).

The targeting efficacy of systemically delivered atelocollagen/siRNA complexes has been assessed in other studies. In a mouse model of bone metastatic human prostate cancer cells stably expressing luciferase in mouse thorax, jaws and/or legs, i.v. injection of GL3 luciferase siRNAs complexed with atelocollagen resulted in rapid downregulation of luciferase activity (Takeshita *et al*. 2005). The 'enhancer of zeste homolog 2' (EZH2) and phosphoinositide 3'-hydroxykinase p110-α-subunit (p110α) have been described as candidate targets for inhibition of bone metastasis. In the same system, reduced metastatic tumor growth in bone tissues was observed upon i.v. injection of atelocollagen/siRNA complexes targeting EZH2 and p110α (Takeshita *et al*. 2005).

Another study employed a mouse model for pulmonary metastases following tail vein injection of melanoma cells. Upon i.p. injection of siRNAs targeting tissue factor, an inhibitory effect on metastasis formation was observed, indicating that tissue factor has a crucial function in promoting lung tumor metastasis (Amarzguioui *et al*. 2006). The fact that reduced metastasis formation was observed independently of the time point of siRNA treatment (i.e., before or after cell injection) may also indicate that siRNA-mediated gene targeting does not represent an only short-term effect.

Nevertheless, other studies employed regular siRNA treatment. Ocker *et al*. described that i.p. injection of siRNAs targeting bcl-2 inhibited expression of the target gene and led to antiproliferative and proapoptotic effects in pancreatic tumor xenografts when nude mice were treated daily for 24 days. However, biodistribution assays also revealed that siRNAs were quickly distributed to all organs examined and excreted via liver and kidney (Ocker *et al*. 2005). Likewise, bcl-2-targeting was explored in a mouse model of liver metastasis. Upon i.v. administration of siRNAs complexed with the novel cationic liposome LIC-101, a strong anti-tumoral effect was observed. Delivery of siRNAs to tumor cells in the mouse liver was dependent on complexation with LIC-101 and absent without complexation (Yano *et al*. 2004). In the same study, LIC-101/siRNA complexes targeting bcl-2 were injected around subcutaneous prostate cancer xenografts in mice. Again, tumor growth inhibition was observed (Yano *et al*. 2004).

Another novel cationic liposome, based on a synthetic cationic cardiolipin analogue (CCLA), was employed by Chien *et al*. and displayed low toxicity. In SCID mice bearing human breast tumor xenografts, upon targeting of c-raf through CCLA-complexed, specific siRNAs, a 73% reduction of tumor growth was observed as compared to free anti-c-raf siRNAs (Chien *et al*. 2005).

Breast cancer xenografts as tumor model in nude mice were also employed for siRNA-mediated targeting of Ras homologous A (Rho A) which has been described as an indicator of poor prognosis due to increased tumor cell proliferation, invasion and tumor angiogenesis. I.v. injection of Rho A-specific siRNAs complexed with chitosan-coated polyisohexylcyanoacrylate (PIHCA) nanoparticles (Maksimenko *et al*. 2005) led to a marked >90% inhibition of tumor growth and the occurrence of necrotic areas resulting from inhibition of angiogenesis. Furthermore, based on body weight gain, biochemical markers of hepatic, renal and pancreatic function, and macroscopic appearance of organs after treatment for 1 month, the absence of non-specific toxic effects was observed (Pille *et al*. 2006).

Orthotopic hepatocellular carcinoma xenografts were also the target organ for siRNA-mediated acid ceramidase silencing. Hydrodynamic i.v. injection of siRNAs led to reduced tumor growth and increased sensitivity towards daunorubicin (Morales *et al*. 2007).

Targeting β-catenin was performed in i.p. injected colon cancer adenocarcinoma cells through i.p. injection of liposome-encapsulated siRNAs. Following-up for a period of 70 days revealed in some mice an increase in survival upon β-catenin siRNA treatment (Verma *et al*. 2003). Nogawa *et al*. exploited the fact that in patients with bladder cancers the expression of high levels of polo-like kinase-1 (PLK-1) is a negative prognostic marker. Targeting PLK-1 through intravesical injection of siRNAs in cationic liposomes prevented the growth of bladder cancer in an orthotopic bladder cancer mouse model (Nogawa *et al*. 2005). Likewise, focal adhesion kinase (FAK) has been shown previously to play a critical role in ovarian cancer cell survival and metastasis and provided the basis for a recent study on targeting FAK in ovarian cancer xenografts in nude mice. Treatment with FAK-targeting siRNAs in the neutral liposome DOPC resulted in reduction of mean tumor weight by 44% to 72% and in even stronger effects upon combined treatment with cisplatin or docetaxel. With the latter treatment regimen, decreases in microvessel density, in VEGF expression and in MMP-9 expression were observed, as well as increased apoptosis of tumor-associated endothelial cells and tumor cells. Thus, this study also demonstrates additive effects when combining siRNA-mediated targeting and standard treatment with a cytostatic agent (Halder *et al*. 2006).

Ovarian cancer xenografts were also used as a model system for targeting the oncoprotein EphA2. DOPC-encapsulated specific siRNAs showed highly efficient reduction of *in vivo* EphA2 expression, and treatment of tumor-bearing mice for three weeks with DOPC/EphA2 siRNAs resulted in decreasced tumor growth. Additionally, the combination with paclitaxel displayed an additive treatment effect (Landen *et al*. 2005), again indicating the therapeutic benefit of combining gene targeting with a cytostatic agent.

In other studies, the delivery of siRNAs to tumor endothelial cells, rather than tumor cells, was explored for anti-angiogenic intervention. Santel *et al*. demonstrated that siRNAs formulated in liposomes, but not naked siRNAs, are delivered to tumor endothelial cells and that this approach allows targeting of the tumor suppressor PTEN in liver and tumor endothelial cells. Furthermore, in two different tumor xenograft models, systemic administration of liposomally formulated siRNAs targeting CD31 inhibited tumor growth (Santel *et al*. 2006). Targeting of CD31 or Tie2 was also described in another paper by the same group, as demonstrated by reduced mRNA and protein levels *in vivo* (Santel *et al*. 2006).

The antibody-mediated *in vivo* delivery of siRNAs via cell surface receptors was explored by designing a protamine-antibody fusion protein for the uptake of siRNAs into HIV-infected or envelope-transfected cells. In subcutaneous melanoma xenografts derived from envelope-expressing B16 cells, siRNA-mediated targeting of c-myc, MDM2 and VEGF resulted in inhibition of tumor growth (Song *et al*. 2005). In the same study, a fusion protein consisting of protamine and a HER-2 (c-erbB2) single-chain antibody (scFv) was constructed which allowed the specific delivery of siRNAs into HER-2-expressing tumor cells. S.c. melanoma xenografts were also the target in a more recent paper. Electroporation-mediated *in vivo* transfection of specific siRNAs led to downregulation of microphthalmia-associated trans-

cription factor and displayed anti-tumorigenic effects due to increased apoptosis (Nakai *et al*. 2007).

Other studies exploited HER-2, which is overexpressed and serves as a negative prognostic factor in many tumors, as target molecule for downregulation rather than recognition molecule for ligand-mediated siRNA uptake. Upon i.p. injection of PEI-complexed siRNAs, the efficient delivery of intact siRNA molecules into s.c. ovarian carcinoma xenografts was observed (Urban-Klein *et al*. 2005). In contrast, the delivery of naked siRNAs was poor, as already described in other studies (see above). Treatment of tumor-bearing mice with PEI-complexed HER-2-specific siRNAs resulted in a marked downregulation of HER-2 expression on mRNA and protein levels as well as in a significant anti-tumorigenic effect, characterized by a ~50% tumor growth reduction as compared to the control group (treated with PEI-complexed non-specific siRNA or naked HER-2 specific siRNA, or untreated) (Urban-Klein *et al*. 2005).

Recently, the use of PEI-complexed siRNAs was extended to other tumor xenograft models and administration modes. Pleiotrophin (PTN) has been shown to be rate-limiting for tumor growth in several tumors including glioblastoma (Grzelinski *et al*. 2005) indicating that it may represent an attractive target. In a s.c. glioblastoma xenograft model, PEI/siRNA complexes targeting PTN were i.p. or s.c. (at a site distant from the tumor) injected which again resulted in the delivery of intact siRNAs. Concomitantly, both approaches led to the downregulation of PTN gene expression, as determined on mRNA and protein levels, and significantly reduced tumor growth. The absence of a measurable induction of siRNA-mediated immunostimulation as well as direct comparison with PEI-complexed non-specific siRNAs also indicates the absence of non-specific effects. Furthermore, in a clinically more relevant orthotopic glioblastoma mouse model, intracranial injection of the PTN-specific PEI/siRNA complexes resulted in a marked reduction of tumor growth compared to PEI-complexed non-specific siRNAs or buffer control. Immunohistochemical analysis showed decreased PTN levels thus confirming successful gene targeting and also revealed that the observed antitumorigenic effects were due to reduced tumor cell proliferation (Grzelinski *et al*. 2006). Keeping in mind that in glioblastoma patients the direct injection of therapeutically relevant nucleic acids (i.e., antisense oligonucleotides) into the brain is already in clinical phase II (Schlingensiepen *et al*. 2006), the application of PEI-complexed siRNAs targeting glioblastoma-relevant gene products may in fact represent a feasible approach and a promising strategy. In both above-mentioned studies, commercially available jetPEI was used which displayed high efficacy of siRNA protection and delivery, and showed no toxicity at the amounts used (10 ug siRNA per injection with 2-3 injections per week). Additionally, in another study anti-tumorigenic effects of PEI-complexed siRNAs were observed in s.c. pancreatic carcinoma xenografts after intratumoral injection of PEI/siRNAs targeting mutant K-ras (Zhu *et al*. 2006).

As described above, the PEI-mediated delivery of siRNAs has been extended towards other low-molecular weight PEIs and/or chemical modifications including PEGylation or coupling of specific ligands. The administration of siRNAs targeting VEGF, complexed with a newly developed low-molecular-weight PEI, displayed marked anti-tumorigenic effects in a s.c. prostate carcinoma xenograft model in the mouse, based on the robust downregulation of VEGF expression on mRNA and protein levels. Again, local or systemic toxicity was low or absent with the amounts used for efficient gene targeting (Hobel and Aigner, unpublished data). In a very recent study, a water soluble lipopolymer (WSLP), based on a branched 1800 Da PEI as cationic headgroup, was employed. Intratumoral injection of VEGF siRNAs, complexed with WSLP or PEI, in s.c. PC-3 tumor xenografts led to reduced tumor growth (Kim *et al*. 2007).

Schiffelers *et al*. demonstrated that nanoparticles comprising of PEGylated PEIs with the peptide Arg-Gly-Asp coupled to the distal end of the PEG chains showed increased specificities for tissues expressing integrins and allowed the targeting of the tumor neovasculature. These ligand-targeted, sterically stabilized nanoparticles were used for the delivery of siRNAs inhibiting the VEGF receptor-2 (VEGFR-2). I.v. administration into tumor bearing mice led to selective uptake into s.c. tumor xenografts, downregulation of the target protein within the tumor and inhibition of both tumor growth and tumor angiogenesis (Schiffelers *et al*. 2004). Likewise, higher tumor selectivity was also achieved by encapsulation of siRNAs into a liposomal nanoplex with an anti-transferrin receptor single-chain antibody fragment (TfRscFv; see above). In human breast carcinoma xenografts in mice, delivery of encapsulated siRNA targeting HER-2 upon repeated i.v. treatment led to inhibition of HER-2 expression and induced apoptosis (Hogrefe *et al*. 2006).

OTHER PATHOLOGIES

Many studies on the silencing of pathologically relevant genes have focussed either on local application of formulated or naked siRNAs, or, when applied systemically, on gene-targeting in the liver.

In a study by Zender *et al*. targeting of caspase 8, which is a key enzyme in death receptor-mediated apoptosis, was explored in different mouse models of acute liver failure. Upon systemic application of specific siRNAs, caspase 8 expression in the liver was inhibited. Consequently, Fas (CD95)-mediated apoptosis was prevented, and this protecttion of hepatocytes resulted in the reduction of experimentally induced acute liver damage. Improvement of survival due to siRNA-mediated caspase 8 downregulation was also observed when the treatment was started later during already ongoing acute liver failure, and in an animal model reflecting multiple molecular mechanisms in human acute viral hepatitis (Zender *et al*. 2003). Likewise, in another study Fas was directly targeted to protect mice from liver failure and fibrosis in two models of autoimmune hepatitis. Intravenous administration of Fas siRNAs 2 d before induction of hepatitis by ConA abrogated hepatocyte necrosis and inflammatory infiltration and markedly reduced serum concentrations of transaminases. When the treatment was started only one week after initiation of ConA injections, protection from liver fibrosis was observed. Finally, in a more fulminant hepatitis model, an increase in survival rate upon Fas silencing was observed (Song *et al*. 2003).

Likewise, since Fas-mediated apoptosis has been suggested to contribute to tubular cell death as well, Fas targeting was also performed in kidney after renal ischemia-reperfusion injury induced by clamping of the renal artery. The aim of the study was to protect mice from acute renal failure. Pretreatment of mice with Fas siRNAs by different systemic treatment regimens led to reduced tubular apoptosis and less atrophy and hyaline damage as well as to a higher survival rate. An increase in survival rate was also observed upon postischemic injection of siRNAs through the renal vein (Hamar *et al*. 2004). Finally, siRNA-mediated Fas targeting was employed in a hemorrhage-induced septic acute lung injury model through intranasal application. Upon intratracheal administration of Fas siRNAs to mice subjected to hemorrhagic shock and sepsis, reduced levels of interleukins IL-6, -10, -12, TNF-α, interferon-γ and caspase-3 activity were observed. This effect was paralleled by the preservation of the alveolar architecture and reduction of pulmonary epithelial apoptosis and neutrophil infiltration, indicating the feasibility of intrapulmonary administration of siRNAs and the pathophysiological relevance of Fas targeting in this model (Perl *et al*. 2005). In contrast, targeting heme oxygenase-1 (HO-1), a cytoprotective enzyme system in various injury models, led to increased Fas expression and caspase-3 activity. In this study, lung-specific siRNA delivery was achieved through intranasal administration, resulting in enhanced apoptosis in endothelial cells and in the

mouse lung during lung ischemia-reperfusion injury (Zhang *et al*. 2004). Likewise, in a recent study VEGF transgenic mice were treated with HO-1 siRNAs and exposed to hyperoxia. The observed increased injury indicated that VEGF-induced heme oxygenase-1 confers cytoprotection from lethal hyperoxia *in vivo* (Siner *et al*. 2007). Intratracheal instillation was also performed with siRNAs targeting keratinocyte-derived chemokine (KC) or macrophage-inflammatory protein-2 (MIP-2). Local silencing following traumatic shock/hemmorhage led to suppressed signaling for polymorphonuclear neutrophil (PMN) influx in the lung, thereby reducing acute lung injury (ALI) associated with a secondary septic challenge (Lomas-Neira *et al*. 2005). Recently, silencing of TREM-1 (triggering receptor expressed on myeloid) in a fecal peritonitis mouse model was described. A blunted inflammatory response, impaired bacterial clearance and increased mortality was observed (Gibot *et al*. 2007).

Systemic administration through hydrodynamic injection has been performed for targeting the organic anion transporter 3 (OAT3) in brain capillary endothelial cells. Upon OATC3 downregulation in the blood-brain barrier, reduced brain-to-blood transport of an OAT3 substrate was observed (Hino *et al*. 2006). Other studies in the brain rely on intraventricular/intrathecal modes of administration. Intraventricular infusion of siRNAs targeting the serotonin transporter (SERT) led to reduction of SERT expression in the raphe nuclei. Comparable to the established anti-depressant citalopram, the observed downregulation of SERT-binding sites resulted in an antidepressant-related behavioral response (Thakker *et al*. 2005). Likewise, targeting the dopamine transporter (DAT) through infusion of siRNAs into the ventricular system resulted in significant DAT downregulation and also elicited a temporal hyperlocomotor response. This effect was again similar to the effect observed upon infusion of a pharmacologically selective DAT inhibitor (Thakker *et al*. 2004). In rats, intrathecal infusion of siRNAs targeting the pain-related cation-channel P2X3 through mini-osmotic pumps led to decreased P2X3 mRNA in the dorsal root ganglia and reduced protein levels in the dorsal horn of the spinal cord. While non-specific effects were absent, diminished pain responses in models of both agonist-evoked pain and chronic neuropathic pain were observed (Dorn *et al*. 2004). siRNAs complexed with polyethylenimine were used for intrathecal injection in a study on the targeting of the N-methyl-D-aspartate (NMDA) receptor subunit protein NR2B in a rat model. Primary goal of the treatment was again the modulation of pain which was achieved as indicated by the abolishment of formalin-induced pain behaviors following NR2B downregulation. This study also emphasizes the advantage of gene-targeting approaches, showing high specificity even for subtypes of receptors, over antagonists with often somewhat less specific binding characteristics (Tan *et al*. 2005). Another study on the intrathecal delivery of siRNAs also emphasizes the dose-dependence of specific targeting effects. When delta opioid receptor (DOR) siRNAs were formulated with the cationic lipid i-Fect and delivered as repeated daily bolus doses via implanted intrathecal catheters to the lumbar spinal cord of rats, a blockage of DELT antinociception upon DOR downregulation was observed (Luo *et al*. 2005). However, the targeting success was dose-dependent with no effect at the lowest (0.5 μg) daily dose, indicating the presence of a minimum threshold amount of siRNAs necessary for the induction of RNAi. Thus, insufficient siRNA amounts at the site of action may be one explanation for unsuccessful targeting data which, however, are usually not published (see Hassani *et al*. 2005 for an exception).

Alterations of synaptic function were also achieved through direct siRNA injection into the eye. The amyloid precursor protein (APP) has been shown to play a central role in Alzheimer's disease (AD) through accumulation of its cleavage produce, amyloid β peptide, which is thought to lead to synaptic dysfunction representing an early change in AD. Intraocular injection of siRNAs targeting APP led to reduced levels of APP, as well as of amyloid precursor-like protein 2 (APLP2), in retinal terminals in the superior colliculus, resulting in reduced stimulation-induced glucose utilization in functional imaging experiments suggesting alterations of synaptic function (Herard *et al*. 2005).

The feasibility of RNAi-mediated target gene downergulation through subretinal delivery of siRNAs was also demonstrated by blocking the production of endogenous mVEGF, thus inhibiting laser-induced choroidal neovascularization (CNV) in mice (Reich *et al*. 2003). This suggested the use of RNAi strategies in the treatment of retinal diseases, including pathological disorders based on abnormal blood vessel growth. Likewise, reduced ocular neovasularization induced by treatment with proinflammatory CpG oligonucleotides or herpex simplex virus infection was observed upon treatment with siRNAs targeting either VEGFA, VEGFR1, VEGFR2, or a mix of the three. Treatment strategies included the local administration of naked siRNAs or the systemic i.v. injection of siRNAs complexed with PEI-PEG-RGD, and indicated the putative relevance of VEGF pathway-specific siRNAs in the therapy of neovascularization-related eye diseases. This was also confirmed in a study by Shen *et al*. using Sirna-027, a chemically modified siRNA drug targeting VEGFR1. In a CNV model, intravitreous or periocular injection of Sirna-027 led to significant reductions in neovascularization, and intravitreous injection of Sirna-027 reduced retinal neovascularization in mice with ischemic retinopathy (Shen *et al*. 2006). In a clinical study by Sirna Therapeutics employing Sirna-027 for the treatment of age-related macular degeneration (AMD), stabilization and even improvement of visual acuity was observed (Whelan 2005). Additionally, Acuity Pharmaceuticals has introduced the siRNA drug Cand5 targeting VEGF for intravitreal injection in the same disease. Results of a phase I clinical trial indicated adverse effects only related to the route of administration, but not to the drug, and no systemic delivery.

Finally, several studies have demonstrated systemic efficacy of formulated and/or chemically modified siRNAs. Inhibition of intimal hyperplasia is a major obstacle to patency after vein grafting, and the heparin-binding growth factor midkine (MK) has been suggested as a candidate target protein for preventing vein graft failure. In Japanese white rabbits, the perivascular application of atelocollagen/siRNA complexes targeting midkine led to the knockdown of the gradually increasing MK expression after operation, and intimal hyperplasia was reduced. Thus, this strategy may prevent vein graft failure (Banno *et al*. 2006). Likewise, i.v. administration of siRNAs targeting MK led to inhibition of antibody-induced arthritis, indicating the significance of MK as a target molecule for treatment of prevention of rheumatoid arthritis (Yamamoto *et al*. 2006).

Treatment of spontaneously hypertensive rats with liposome-encapsulated siRNAs targeting β1-adrenergic receptor resulted in efficient delivery into various organs including heart, and in significantly lowered blood pressure due to β1-AR downregulation. Concomitantly, pretreatment before induction of myocardial infarction significantly improved cardiac function and thus displayed a protective and therapeutic effect (Arnold *et al*. 2007).

Soutschek *et al*. demonstrated that chemical stabilization and cholesterol-conjugation resulted in markedly improved pharmacological properties of siRNAs *in vitro* and *in vivo*. Consequently, the i.v. injection of apolipoprotein B-specific siRNAs led to decreased apoB mRNA levels in liver and jejunum, reduced apoB plasma levels and reductions in total cholesterol (Soutschek *et al*. 2004). In an alternative approach for targeting the same gene product, ApoB-specific siRNAs were encapsulated in stable nucleic acid lipid particles (SNALPs), liposomal formulations based on lipid and non-lipid components. The i.v. injection of SNALP-encapsulated ApoB-specific siRNAs into cynomolgus monkeys is also the first example of a study on the systemic efficacy of formulated siRNAs in a non-rodent species. A dose-dependent, up to >90% silencing of AOPB mRNA in the li-

ver 48 h after administration, as well as reductions in ApoB protein, serum cholestrol and low-density lipoprotein levels were observed already 24 h after a single siRNA injection. It should be also noted that effects lasted for 11 days, also indicating again a rather long-lasting biological effect (Zimmermann *et al*. 2006).

CONCLUDING REMARKS

Since its introduction less than 10 years ago, RNA interference has gained high acceptance and wide-spread use as an efficacious method for the targeting of any gene of interest. Its success *in vivo*, which may offer novel therapeutic strategies in various diseases, will largely depend on the development of powerful delivery strategies of efficient RNAi-inducing agents like siRNAs, while avoiding off-target and other unwanted side effects. To this end, reagents and strategies are employed which either represent novel approaches or have been described previously for the delivery of other nucleic acids. The choice of the best delivery tool as well as the route of administration will be major determinants of the successful application of siRNAs *in vivo*. While several studies, published on various siRNA delivery systems, show very promising results, their total number is still loo low to assess the functional performance of any individual strategy. Due to the high relevance of RNAi *in vivo*, however, this is likely to change within the next years. Furthermore, several companies have already reported preclinical data on siRNA *in vivo* drug development projects, and first clinical trials are already ongoing or completed.

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